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Research Article

# Microalgae diversity in *Aedes aegypti* larvae guts and breeding sites in Nakhon Si Thammarat, Thailand revealed by light microscopy and metabarcoding

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## Abstract

*Aedes aegypti* poses a significant threat by transmitting diseases like dengue, chikungunya and Zika, leading to high morbidity and mortality worldwide. In recent years, the role of microalgae in mosquito larvicidal activity has gained attention due to their role as a key mosquito nutritional resource. Understanding microalgal communities in water from breeding sites and *Ae. aegypti* larval guts might be beneficial for implementing mosquito control strategies. The purpose of this work was to investigate the diversity of microalgae in the guts and breeding sites of *Ae. aegypti* larvae in Nakhon Si Thammarat, Thailand, using light microscopy and metabarcoding. Results showed a total of 27 algal genera from this study. Ten genera were identified using light microscopy and an additional 21 genera were identified through 23S metabarcoding. Four genera, *Tetradesmus*, *Nannochloropsis*, *Monoraphidium* and *Leptolyngbya*, were detected by both approaches. Two genera, *Tetradesmus* and *Leptolyngbya*, were present in all guts and water samples. Spearman's correlation analysis, alpha diversity and beta diversity demonstrated a high degree of compositional similarity between gut samples and water samples collected from breeding sites. The results indicated that *Ae. aegypti* larvae could consume nearly all the microalgae in their breeding habitat. Information provided in this study would help choose microalgae that could potentially be a biocontrol agent for further development of eco-friendly products for the future management of mosquito larvae.

**Key words:** *Aedes aegypti*, breeding site, diversity, larvae, metabarcoding, microalgae, mosquito, Thailand

## Introduction

*Aedes aegypti* mosquitoes act as vectors of arboviruses across tropical and subtropical regions worldwide (Gwee et al. 2021) causing diseases such as dengue, chikungunya and Zika with high morbidity and mortality (Naslund et al. 2021). The prolonged and extensive application of pesticides for mosquito control, such as synthetic pyrethroids (permethrin and deltamethrin), organochlorines (DDT, lindane and dieldrin), organophosphates (parathion, chlorpyrifos and diazinon), has resulted in the emergence of insecticide resistance and alterations in mosquito feeding behaviour. Additionally, the persistence of pesticide residues in food and the environment give rise to several detrimental impacts on human health, i.e. neurological disorders, endocrine disruption and cancer (Mishra et al. 2012; Viel et al. 2015; Huang et al. 2019; Park et al. 2019; Wongta et al. 2022; Wu et al. 2023; Ahmad et al. 2024; Zhou et al. 2025). Given the rapid resistance of vector mosquitoes to insecticide usage, eco-friendly bioproducts are urgently needed for effective vector control programmes (Jemima and Kumar 2023).

Microalgae are a diverse group of photosynthetic microorganisms that are found in aquatic environments. They occur as single cells, colonies or filaments that become visible to the naked eye during bloom events. Microalgae play pivotal roles in nutrient cycling and ecosystem dynamics (Tufan-Cetin and Cetin 2023). They have recently also gained attention as a potential biological control agent for mosquito larvae, as they are a key mosquito nutritional resource (Fei et al. 2021; Gil et al. 2021; Perumal et al. 2022). Microalgae, such as *Chlorella* sp. and *Arthrospira platensis*, provide essential nutrients, particularly protein, for mosquito larvae (Souza et al. 2019). Ahmad et al. (2004) reported that *Ae. aegypti* fed with *Chlorococcum* UMACC 218 and *Scenedesmus* UMACC 220 experienced delayed pupation and were reduced in body size. Therefore, understanding the relationship between *Ae. aegypti* mosquitoes and microalgae present in their habitats is crucial information for implementing the mosquito control and mitigating the burden of mosquito-borne disease.

Microalgae identification is mainly based on the light microscopic approach and morphological identification keys (Prescott 1964; Bellinger and Sigee 2015). Some microalgae have unique features that allow for easy distinction; however, some species share similar morphological traits at certain life cycle stages, potentially leading to misidentification. Combining morphological observations with molecular techniques, such as DNA sequencing, provides more precise identification by examining the genetic markers of microalgae, allowing for more accurate taxonomic classification and identification results (Manoylov 2014). Recently, amplicon-based metabarcoding has proven to be an efficient tool in analysing complex microbial communities (Handelsman 2004). Metabarcoding targets regions of DNA (barcodes) that have enough variability to distinguish different taxonomic groups, allowing for simultaneous identification of many taxa within the same sample. Metabarcoding is helpful to understand species composition at the community level using mixed bulk samples and reveal “hidden diversity” that might be missed by traditional methods (Taberlet et al. 2012; Bucklin et al. 2016; Trivedi et al. 2016).

In Thailand, most studies regarding microalgae have focused on the diversity, distribution and relationships to the physio-chemical environments (Fu et al. 2021; Prasertsin et al. 2021; Ruen-Pham et al. 2021). Despite this, knowledge regarding

microalgae composition within the gut of mosquito larvae across diverse breeding sites in Thailand remains limited. Therefore, this study aimed to investigate the diversity of gut microalgae of *Ae. aegypti* larvae, using both microscopy and 23S rRNA metabarcoding, across three main breeding sites in Nakhon Si Thammarat, Thailand, which recorded a high number of dengue cases since 2000 (Department of Disease Control, https://ddc.moph.go.th/). The knowledge gained from this study offers potential insights and a solid foundation for developing biological control agents or eco-friendly bioproducts targeting *Ae. aegypti* mosquitoes.

## Materials and methods

### Study location

Sample collection was carried out between September 2022 and March 2023. *Aedes aegypti* breeding sites were selected, based on where dengue haemorrhagic fever (DHF) was endemic. Mueang District was selected since an average of 2,229 dengue cases were reported within the past five years, which was also the highest rank in the Nakhon Si Thammarat Province, Thailand (Department of Disease Control, https://ddc.moph.go.th/). Three study locations were selected (Table 1, Suppl. material 1). A summary of the workflow of this study is shown in Fig. 1.

### Identification of *Ae. aegypti* larvae in the field and collection of samples

Larvae of *Ae. aegypti* were identified in the field, based on the characteristics of larval locomotion and siphon shape according to pictorial keys (Rueda 2004). At each of the three study sites, three larval samples and three water samples were collected from sub-sites that were at least 500 m apart. For example, at the MP location, three larval samples (MPL1, MPL2 and MPL3) and three water samples (MPW1, MPW2 and MPW3) were obtained (Table 1). At each sub-site, three to five containers were selected, with each container containing at least 500 ml of water and more than 70 third- and fourth-instar *Ae. aegypti* larvae. Then, the larvae and water from these three to five selected containers were pooled to collect one larval sample (a pool of 300–400 third- and fourth-instar larvae) and one water sample (a pooled 500 ml of water). Therefore, a total of nine larval and nine water samples were obtained and used for further analyses. During sampling, the water samples and larvae were collected from the surface of the containers to prevent contamination from soil or plant microorganisms.

### Temperature and pH of water in the breeding sites

The temperature and pH of water from the breeding sites were measured, using a parameter analyser (HQ 40d multi, HACH), as described by Elnaggar and El-Alfy (2016).

**Table 1.** Study locations in Nakhon Si Thammarat Province, Thailand and the sample ID.

| Study location | Coordinates | Sample ID for larval guts | Sample ID for water from breeding site |
|---|---|---|---|
| Mum Pom Temple communities (MP) | 8°25'38.1"N, 99°57'56.9"E | MPL1, MPL2, MPL3 | MPW1, MPW2, MPW3 |
| Bun Na Rop Temple communities (BN) | 8°25'06.4"N, 99°58'05.6"E | BNL1, BNL2, BNL3 | BNW1, BNW2, BNW3 |
| Ban Na Kian communities (NK) | 8°26'41.2"N, 99°54'07.1"E | NKL1, NKL2, NKL3 | NKW1, NKW2, NKW3 |

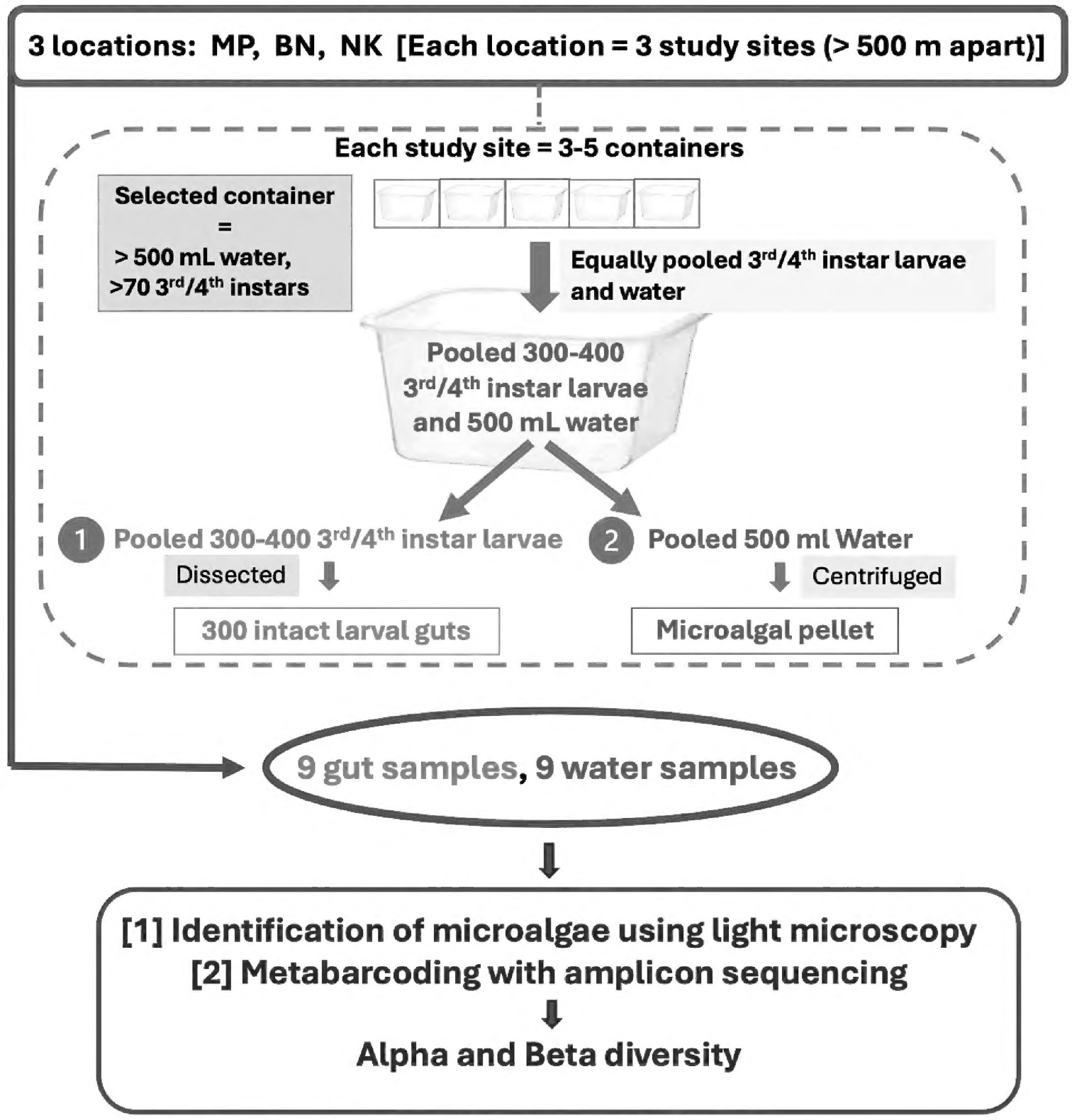


**Figure 1.** Summary of the workflow for this study.

### Isolation of microalgae from a water sample

To isolate microalgae from the water sample, 500 ml of pooled water from each site was repeatedly transferred into 50 ml sterile conical tubes and centrifuged three times at 4,500 rpm for 10 min at 25 °C (Garros et al. 2008). Then, the pellets were re-suspended in 200 µl of sterile phosphate buffered saline (PBS) in a 1.5 ml sterile microcentrifuge tube. The re-suspended sample was divided equally into two sub-samples for microscopic examination (~ 100 µl) and genomic DNA extraction (~ 100 µl). The sample for the microscopic examination was kept in a 1.5 ml sterile tube containing a small volume of Bold's Basal Medium (BBM) at 6–8 °C, while the sample for DNA extraction was stored at -80 °C less than 48 h before extraction to prevent DNA degradation.

### Dissection of larval gut and isolation of microalgae from gut contents of *Ae. aegypti* larvae

To collect 300 intact larval guts, the third- and fourth-instar larvae of *Ae. aegypti* from each site were used. Before dissection, larvae were surface sterilised for 30 seconds in a microcentrifuge tube containing 500 µl of 70% ethanol, followed by twice

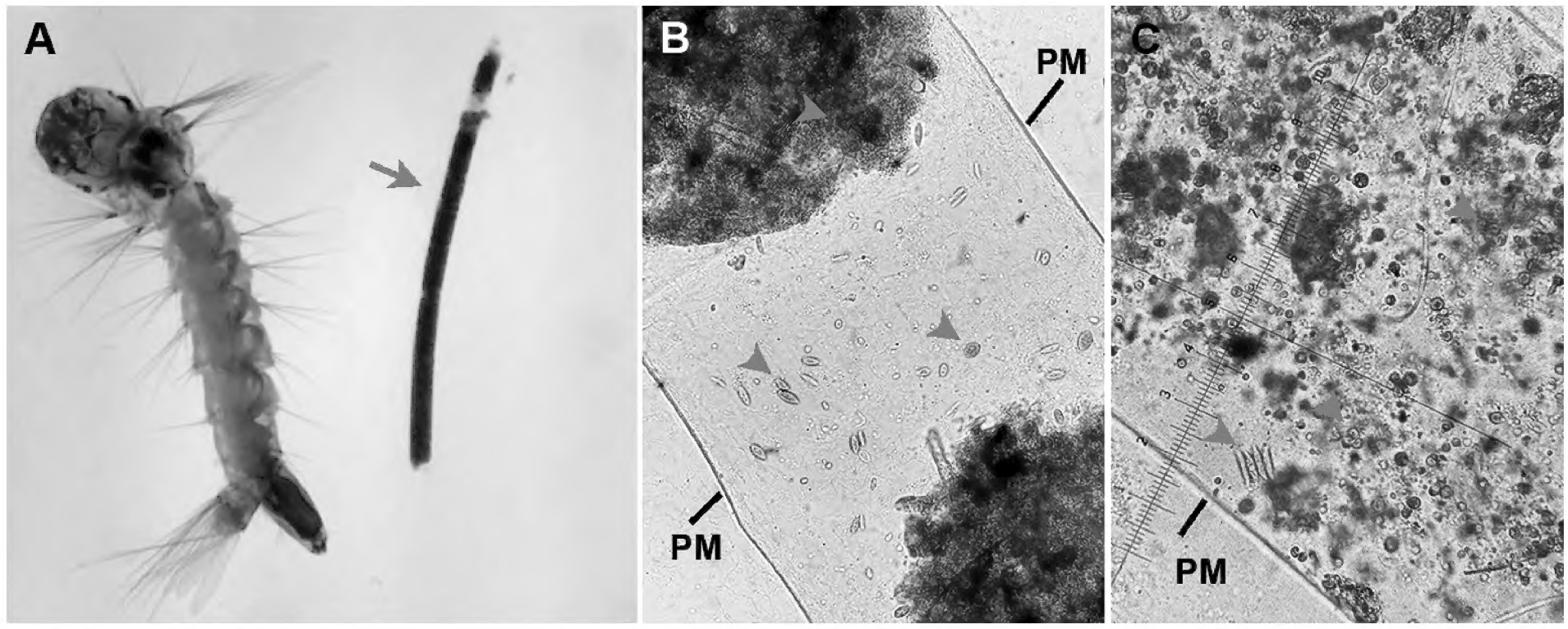


**Figure 2.** *Aedes aegypti* larval gut with intact peritrophic membrane (PM) and the microalgae present within the larval guts. Dissection of *Ae. aegypti* larval gut with intact PM (red arrow) (**A**). The microalgae present within the larval guts (red arrow heads) (**B, C**).

rinsing in 500 µl of PBS. Larval guts were dissected carefully with a sterile needle under a stereo-microscope (Olympus SZ2-ILST, Olympus Corp., Tokyo, Japan). The dissection of 300 larvae was performed in the same day. To minimise contamination, only the gut contents with intact peritrophic membrane were used in this study (Fig. 2). The gut contents of 300 larvae from the same site were pooled into a 1.5 ml sterile tube containing 200 µl of sterile PBS and homogenised using a sterile pestle. Each sample was divided equally into two sub-samples as mentioned above. The sample for microscopic examination was kept in a 1.5 ml-sterile tube containing a small volume of BBM at 6–8 °C, whereas the sample for DNA extraction was stored at -80 °C for less than 48 h before extraction to prevent DNA degradation.

## Morphological identification of microalgae

Microalgae isolated from water and gut contents were examined under a microscope (ECLIPSE E100, Nikon Corporation, Tokyo, Japan) and morphologically identified using a freshwater algae identification key (Prescott 1964; John et al. 2002; Komárek and Anagnostidis 2005; Komárek et al. 2014; Wehr et al. 2015; Cho and Lee 2024).

## Genomic DNA extraction of microalgae

The total genomic DNA of the microalgae was extracted from the water and gut contents of *Ae. aegypti* larvae using a PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), according to the manufacturer's instructions. DNA concentration of each sample was measured by a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

## Genetic marker amplification and bioinformatics

In this study, one group of genetic markers, LSU rDNA (Large Subunit Ribosomal DNA) of 23S rDNA, a genetic marker found in all microorganisms that perform

photosynthesis, was used to identify the microalgae from the breeding sites and larval gut (Djemiel et al. 2020; Saraphol et al. 2024). Briefly, 23S metabarcoding with amplicon sequencing library preparation was performed by amplifying 23S rDNA using the forward (p23StV_f1: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGACAGAAAGACCCTATGAA) and reverse primers (p23SrV_r1: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCAGCCTGTTATCCCTAGAG), with modification according to the manual of Illumina MiSeq, of which underlined sequences were overhang adapter sequences (Sherwood and Presting 2007; https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html), targeting eukaryotic algae and cyanobacteria. Herein, 2X sparQ HiFi PCR Master Mix (QuantaBio, USA) was used. The expected length of the fragment was approximately 500 bp. Amplification was done with an initial denaturation at 98 °C for 2 min, followed by 30 cycles of denaturation at 98 °C for 20 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s, followed by a single-step final extension at 72 °C for 1 min. Subsequently, the 23S amplicons were purified with sparQ Puremag Beads (QuantaBio, USA) and indexed using 2.5 µl of each Nextera XT index primer in a 50 µl PCR reaction, followed by 10 cycles of the previously described PCR conditions. After that, PCR products were cleaned, pooled and diluted to a loading concentration of 4 pM. Cluster generation and 250-bp paired-end sequencing were carried out on an Illumina MiSeq at the Omics Sciences and Bioinformatics Centre, Chulalongkorn University, Bangkok, Thailand.

## Bioinformatics

Bioinformatics analysis was conducted using QIIME 2 version 2023.9 (Bolyen et al. 2019). Raw sequence data were demultiplexed and quality filtered with the q2-demux plugin, followed by denoising with DADA2 (Callahan et al. 2016) through the q2-dada2 plugin. Amplicon sequence variants (ASVs) with a total frequency of fewer than 50 reads were filtered out. Taxonomy was assigned to ASVs using the q2-feature classifier (Bokulich et al. 2018), with the classify-sklearn naïve Bayes taxonomy classifier, based on the µgreen-db r1.1_2019_09_06 reference database, which includes 2,326 plastid 23S rRNA reference sequences (1,388 from Eukaryota and 938 from Bacteria) (Djemiel et al. 2020). The samples were restricted to between 16,447 and 17,212 sequences per sample to represent equal sampling efforts amongst the samples prior to visualisation of relative abundances and calculation of diversity indices. Data were visualised using the “ggplot2” package version 3.5.1 in R version 4.4.1 (R Core Team 2024), unless stated otherwise (Wickham 2016).

Relative abundance was calculated by dividing the number of reads of each taxon by the total number of sequences in a sample. The calculated relative abundances were displayed for all 25 identifiable genera using a stacked bar chart and a heatmap. Since the relative abundance data did not follow a normal distribution, Spearman’s correlation test (Hollander and Wolfe 1973) was used to assess whether algal relative abundances in water and larvae were correlated, using the “cor.test” function in the “stats” package (R Core Team 2024).

### Alpha diversity of microalgal community

All subsequent analyses on alpha and beta diversity were performed in R version 4.4.1 (R Core Team 2024). For each sample, the ASVs with taxonomic assignments at the OTU level and their abundances were used to calculate OTU richness (number of OTU), Chao 1, Shannon's Index and Simpson's Index, using the "vegan" package version 2.6–6.1 (Oksanen et al. 2024). We performed a paired t-test to determine whether the alpha diversity differed between the communities in the larval gut and the water from the same breeding site, using the "stats" package version 4.4.1 (R Core Team 2024). The alpha of 0.05 was used as a threshold for significant results for two-tailed p-values.

### Beta diversity of microalgal community

The Bray-Curtis dissimilarity index was calculated to examine the beta diversity of the microalgal communities using the "vegdist" function in the "vegan" package version 2.6–6.1 (Oksanen et al. 2024). The resulting distance matrix was subjected to the Principal Coordinate Analysis (PCoA) to visualise the compositional differences amongst the samples, using the "autoplot" function in the "ggfortify" package version 0.4.17 (Tang et al. 2016). To determine whether taxon composition differed amongst the sources (gut vs. water) and the locations, we performed a Permutational Multivariate Analysis of Variance (PERMANOVA, Anderson (2005)) using the Bray-Curtis distance matrix and the "adonis2" function in the "vegan" package version 2.6–6.1 (Oksanen et al. 2024). The number of permutations was set to 9,999 and p-values were calculated to assess significance for each term (sources, locations and their interaction). The alpha of 0.05 was used as a threshold for significant results for the p-values.

## Results

### Temperature and pH of water in the breeding site

In this study, samples in each breeding site were collected from tyres, plastic containers, ceramic vases and/or glass bottles, respectively. The water temperature ranged from 25.6 °C to 29.2 °C at MP, 26.0 °C to 32.1 °C at BN and 25.6 °C to 33.5 °C at NK. The pH values ranged from 7.30 to 7.78, 6.98 to 8.34 and 7.07 to 7.61 at MP, BN and NK, respectively.

### Morphological identification of microalgae

Various algal morphotypes were present in the larval habitats and guts. These included unicellular, colonial and filamentous cyanobacteria, green algae and diatoms. Across all guts and water samples, at least ten genera were identifiable by their morphology using light microscopy. The known microalgal genera included *Ankistrodesmus*, *Bracteacoccus*, *Chloromonas*, *Eudorina*, *Leptolyngbya*, *Monoraphidium*, *Nannochloropsis*, *Pediastrum*, *Tetradesmus* and *Selenastrum* (Fig. 3).

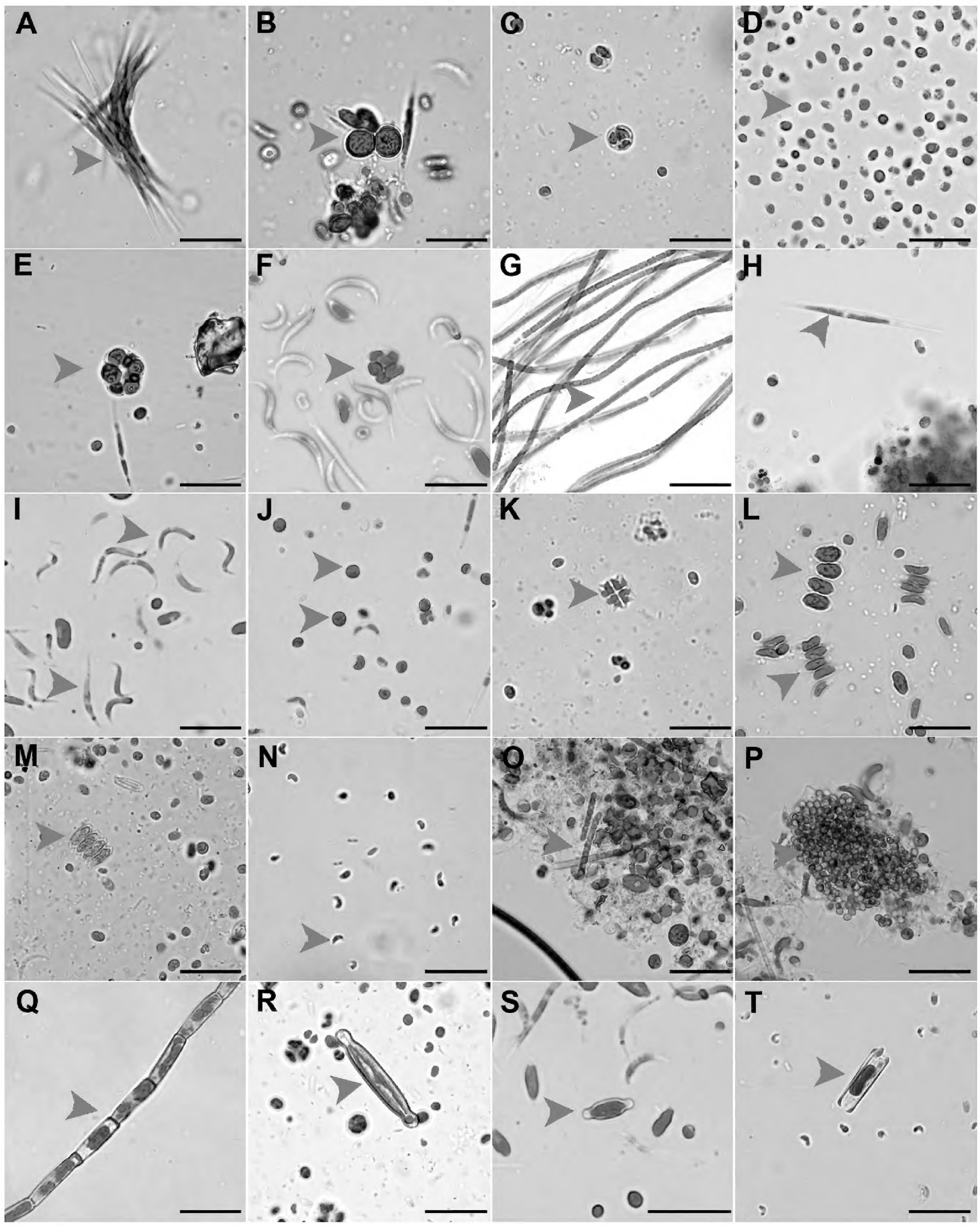


**Figure 3.** Representative microalgae collected from the larval gut and breeding sites, morphologically identified under compound light microscopy to genus level. *Ankistrodesmus* (**A**), *Bracteacoccus* (**B, C**), *Chloromonas* (**D**), *Eudorina* (**E, F**), *Leptolyngbya* (**G**), *Monoraphidium* (**H, I**), *Nannochloropsis* (**J**), *Pediastrum* (**K**), *Tetradesmus* spp. (**L, M**), *Selenastrum* (**N**), unidentifiable cyanobacteria (**O, P**), filamentous green alga (**Q**) and unidentifiable diatoms (**R, S, T**). Bar = 20 micrometres.

## Metabarcoding using 23S rDNA amplicon sequencing of microalgal communities

We successfully obtained 23S rDNA amplicons from 18 examined microalgal communities, including nine from larval guts and nine from breeding sites. All the raw data were deposited in NCBI SRA BioProject PRJNA1182502,

BioSample SAMN44597993-SAMN44598010 (Suppl. material 2) under Submission ID SUB14847725.

After demultiplexing and quality filtering, a total of 308,514 sequences were obtained, of which 31,447 sequences were unassigned or not able to be identified to the genus level. Some136,547 sequences from the larval guts and 140,520 sequences from the sampled water were similarly identified as members of 25 algal genera and diatoms belonging to Achnanthales.

The abundance of the microalgae isolated from the gut of *Ae. aegypti* larvae and the water from their breeding sites, in terms of the number of amplicons, varied amongst sites ((Fig. 4, Suppl. material 3). The top five most abundant taxa were *Tetradesmus*, *Nannochloropsis*, *Leptolyngbya*, *Parachlorella* and *Monoraphidium*, respectively (Fig. 4, Table 2). Amongst these, two algae, *Tetradesmus* and *Leptolyngbya*, were present in all samples (Table 2).

Most of the algal taxa found in the guts were also present in the water. In addition, a strong positive correlation was observed between the quantity of sequences for each genus in *Ae. aegypti* larvae guts and the corresponding sequence count in their breeding site water samples (Spearman's correlation, $\rho = 0.84$, $P < 0.001$) (Fig. 5).

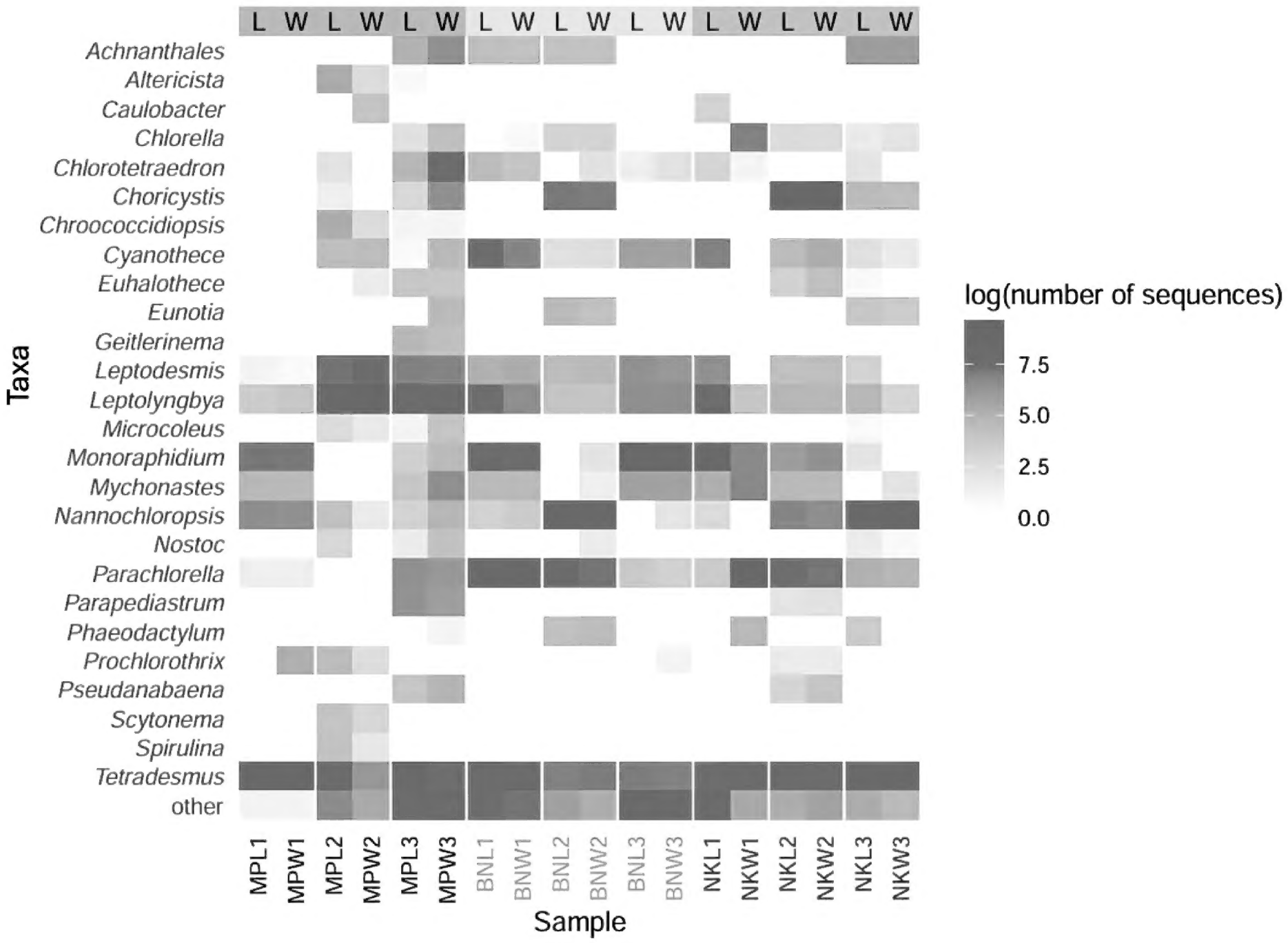


**Figure 4.** A heatmap of the relative abundances of microalgal taxa in the samples from the gut of *Ae. aegypti* larvae and water from the breeding sites of the three locations. X axis represents samples and Y axis represents microalgal genera. The colour scale on the right indicates the scale for the logarithm of the number of sequences. MP, BN and NK are the names of the sampling areas. "L" represents the larval gut and "W" represents the water from breeding sites.

**Table 2.** Relative abundance of the top six microalgal genera in guts and breeding sites of *Ae. aegypti* larvae analysed by metabarcoding with amplicon sequencing.

| Study site | No. | Abundance of microalgae | | | | | |
|---|---|---|---|---|---|---|---|
| | | Guts | | | Water | | |
| | | Microalgae | Number of Reads | % | Microalgae | Number of Reads | % |
| MP1 | 1 | *Tetradesmus* | 15036 | 87.36 | *Tetradesmus* | 15002 | 87.50 |
| | 2 | *Monoraphidium* | 1275 | 7.41 | *Monoraphidium* | 1252 | 7.30 |
| | 3 | *Nannochloropsis* | 754 | 4.38 | *Nannochloropsis* | 578 | 3.37 |
| | 4 | *Mychonastes* | 106 | 0.62 | *Prochlorothrix* | 165 | 0.96 |
| | 5 | *Leptolyngbya* | 34 | 0.20 | *Mychonastes* | 95 | 0.55 |
| | 6 | *Parachlorella* | 3 | 0.02 | *Leptolyngbya* | 48 | 0.28 |
| MP2 | 1 | *Leptolyngbya* | 9875 | 60.04 | *Leptolyngbya* | 13524 | 81.09 |
| | 2 | *Tetradesmus* | 3608 | 21.94 | *Leptodesmis* | 2262 | 13.56 |
| | 3 | *Leptodesmis* | 1469 | 8.93 | *Tetradesmus* | 452 | 2.71 |
| | 4 | *Altericista* | 207 | 1.26 | *Cyanothece* | 80 | 0.48 |
| | 5 | *Chroococcidiopsis* | 181 | 1.10 | *Caulobacter* | 59 | 0.35 |
| | 6 | *Prochlorothrix* | 82 | 0.50 | *Scytonema* | 19 | 0.11 |
| MP3 | 1 | *Tetradesmus* | 8324 | 48.90 | *Leptolyngbya* | 4163 | 25.19 |
| | 2 | *Leptolyngbya* | 2956 | 17.36 | *Chlorotetraedron* | 2037 | 12.33 |
| | 3 | *Leptodesmis* | 1049 | 6.16 | *Tetradesmus* | 1884 | 11.40 |
| | 4 | *Parachlorella* | 590 | 3.47 | *Leptodesmis* | 922 | 5.58 |
| | 5 | *Parapediastrum* | 527 | 3.10 | *Mychonastes* | 655 | 3.96 |
| | 6 | Achnanthales | 146 | 0.86 | Achnanthales | 617 | 3.73 |
| BN1 | 1 | *Parachlorella* | 5915 | 34.54 | *Parachlorella* | 8447 | 49.93 |
| | 2 | *Monoraphidium* | 2802 | 16.36 | *Tetradesmus* | 3106 | 18.36 |
| | 3 | *Tetradesmus* | 2502 | 14.61 | *Monoraphidium* | 1806 | 10.68 |
| | 4 | *Cyanothece* | 2019 | 11.79 | *Cyanothece* | 932 | 5.51 |
| | 5 | *Leptolyngbya* | 1685 | 9.84 | *Leptolyngbya* | 685 | 4.05 |
| | 6 | *Leptodesmis* | 153 | 0.89 | *Leptodesmis* | 187 | 1.11 |
| BN2 | 1 | *Nannochloropsis* | 11362 | 67.84 | *Nannochloropsis* | 12335 | 73.58 |
| | 2 | *Parachlorella* | 2422 | 14.46 | *Parachlorella* | 1454 | 8.67 |
| | 3 | *Choricystis* | 1224 | 7.31 | *Tetradesmus* | 1194 | 7.12 |
| | 4 | *Tetradesmus* | 1028 | 6.14 | *Choricystis* | 1179 | 7.03 |
| | 5 | *Eunotia* | 95 | 0.57 | *Leptodesmis* | 106 | 0.63 |
| | 6 | *Leptodesmis* | 84 | 0.50 | *Phaeodactylum* | 81 | 0.48 |
| BN3 | 1 | *Monoraphidium* | 8807 | 51.72 | *Monoraphidium* | 9139 | 53.56 |
| | 2 | *Tetradesmus* | 1147 | 6.74 | *Tetradesmus* | 1020 | 5.98 |
| | 3 | *Leptolyngbya* | 678 | 3.98 | *Leptolyngbya* | 585 | 3.43 |
| | 4 | *Leptodesmis* | 553 | 3.25 | *Leptodesmis* | 510 | 2.99 |
| | 5 | *Cyanothece* | 329 | 1.93 | *Cyanothece* | 285 | 1.67 |
| | 6 | *Mychonastes* | 307 | 1.80 | *Mychonastes* | 283 | 1.66 |
| NK1 | 1 | *Monoraphidium* | 6300 | 37.11 | *Parachlorella* | 11604 | 67.66 |
| | 2 | *Leptolyngbya* | 2146 | 12.64 | *Tetradesmus* | 2427 | 14.15 |
| | 3 | *Tetradesmus* | 2020 | 11.90 | *Chlorella* | 1020 | 5.95 |
| | 4 | *Cyanothece* | 1052 | 6.20 | *Monoraphidium* | 870 | 5.07 |
| | 5 | *Leptodesmis* | 569 | 3.35 | *Mychonastes* | 866 | 5.05 |
| | 6 | *Mychonastes* | 156 | 0.92 | *Phaeodactylum* | 96 | 0.56 |
| NK2 | 1 | *Choricystis* | 11023 | 64.30 | *Choricystis* | 11110 | 65.39 |
| | 2 | *Tetradesmus* | 2436 | 14.21 | *Tetradesmus* | 2033 | 11.97 |
| | 3 | *Parachlorella* | 1731 | 10.10 | *Parachlorella* | 1587 | 9.34 |
| | 4 | *Nannochloropsis* | 941 | 5.49 | *Nannochloropsis* | 782 | 4.60 |
| | 5 | *Monoraphidium* | 370 | 2.16 | *Monoraphidium* | 559 | 3.29 |
| | 6 | *Leptolyngbya* | 125 | 0.73 | *Cyanothece* | 181 | 1.07 |
| NK3 | 1 | *Nannochloropsis* | 10143 | 60.27 | *Nannochloropsis* | 10654 | 62.81 |
| | 2 | *Tetradesmus* | 5567 | 33.08 | *Tetradesmus* | 5519 | 32.54 |
| | 3 | Achnanthales | 417 | 2.48 | Achnanthales | 401 | 2.36 |
| | 4 | *Parachlorella* | 154 | 0.92 | *Parachlorella* | 129 | 0.76 |
| | 5 | *Leptolyngbya* | 130 | 0.77 | *Choricystis* | 73 | 0.43 |
| | 6 | *Choricystis* | 84 | 0.50 | *Eunotia* | 39 | 0.23 |

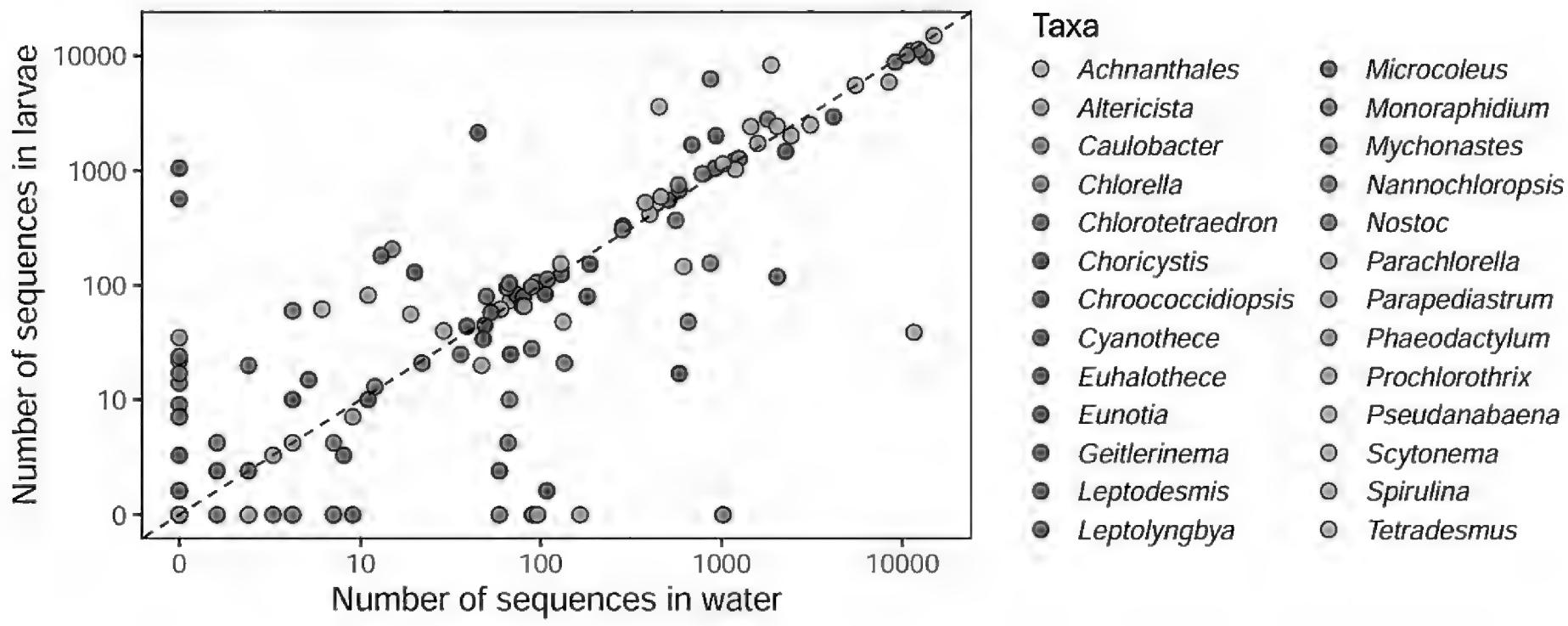


**Figure 5.** Numbers of microalgal sequences in the gut of larvae and water in the same breeding sites of microalgal taxa. The dashed line represents 1:1 ratio between the numbers of sequences in larvae and water.

### Alpha diversity

Alpha-diversity indices, including Shannon's Index, Simpson's Index, Chao1 and OTU richness, of microalgae in the larval guts were not statistically different from those of the water samples from the same breeding sites (paired t-test, $P \geq 0.18$). This suggested the presence of similar algal diversity of the larval guts and the larval habitats. The number of the algal OTUs varied from 22 to 246. The Chao 1 values ranged between 23.50 and 267.48. The Shannon's Index values ranged from 0.69 to 3.59. In addition, the Simpson's Index values ranged from 0.28 to 0.93 (Fig. 6, Suppl. material 4).

### Beta diversity

We assessed the beta diversity of the microalgae in the larval guts and the breeding sites using Bray-Curtis dissimilarity. Results from principal coordinate analysis (PCoA), based on Bray-Curtis dissimilarity, accounted for 29.04% and 17.38%, totalling 46.42% of the total variance. The PERMANOVA analysis showed no significant differences in the composition and community structure of microalgae, based on the source (gut vs. water, $P = 0.99$), sampling locations MP, BN and NK ($P = 0.09$), nor the interaction between source and location ($P = 1.00$; Fig. 7, Suppl. material 5).

## Discussion

To the best of our knowledge, this is the first study to assess the diversity of microalgae present in the guts of *Ae. aegypti* larvae and their breeding sites in Thailand, a region endemic for dengue haemorrhagic fever.

In this study, the pH and temperature of the breeding sites of *Ae. aegypti* in Nakhon Si Thammarat corresponded with previous reports in Guadeloupe, French Guiana and Sri Lanka (Hery et al. 2021; Dalpadado et al. 2022). Generally, *Ae. aegypti* mosquitoes thrive in breeding sites with a pH range of 6 to 9 and temperatures between 26 °C and 32 °C, though they can survive in wider ranges (Ramos et al. 2020; Hery et al. 2021; Dalpadado et al. 2022). Microalgae mainly

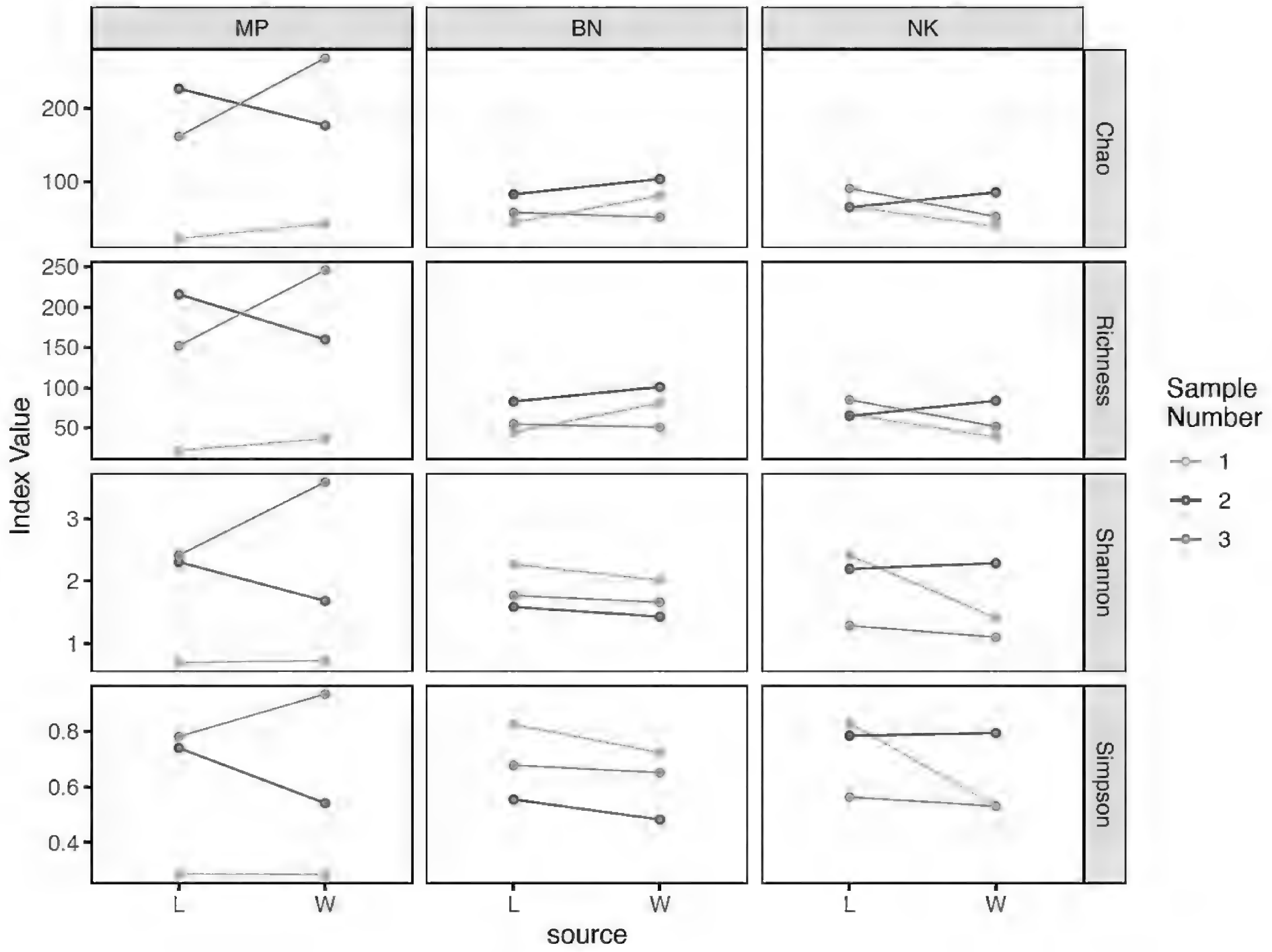


**Figure 6.** Alpha-diversity indices, including Shannon's Index, Simpson's Index, Chao1 and OTU richness, of microalgae from three study areas in Nakhon Si Thammarat, Thailand. MP, BN and NK are the names of the sampling areas. "L" represents the larval gut and "W" represents the water from breeding sites.

prefer a pH range of 6 to 9, but some can tolerate a wider range, including slightly acidic or alkaline conditions. Temperature preferences for microalgae vary by species, but many develop in the 20 °C to 30 °C range (Singh and Singh 2015). These environmental conditions likely supported the algal proliferation found in larval habitats. It suggests that some microalgae and mosquito larvae can co-exist in breeding sites with similar pH and temperature ranges.

No analysis of water samples from containers without larvae was performed in this study since the objective of this study did not involve comparing mosquito breeding sites with and without larvae. In regions where mosquito-borne diseases are endemic, most water in containers consistently harbours larvae. Thus, water samples from the breeding sites (containers) lacking larvae are quite rare. However, this study has provided basic knowledge of the diversity of microalgae found in the guts and breeding sites of mosquitoes as intended.

Here, ten identifiable microalgal genera by the classical light microscopic technique were present in both larval guts and their breeding sites. To achieve a greater comprehension, we employed a metabarcoding technique of 23S rDNA, and an additional 21 algal genera and diatoms belonging to Achnanthales were obtained. Most of the algae found in this study belong to Chlorophyceae, which generally are present in common freshwater habitats in Thailand (Halder et al.

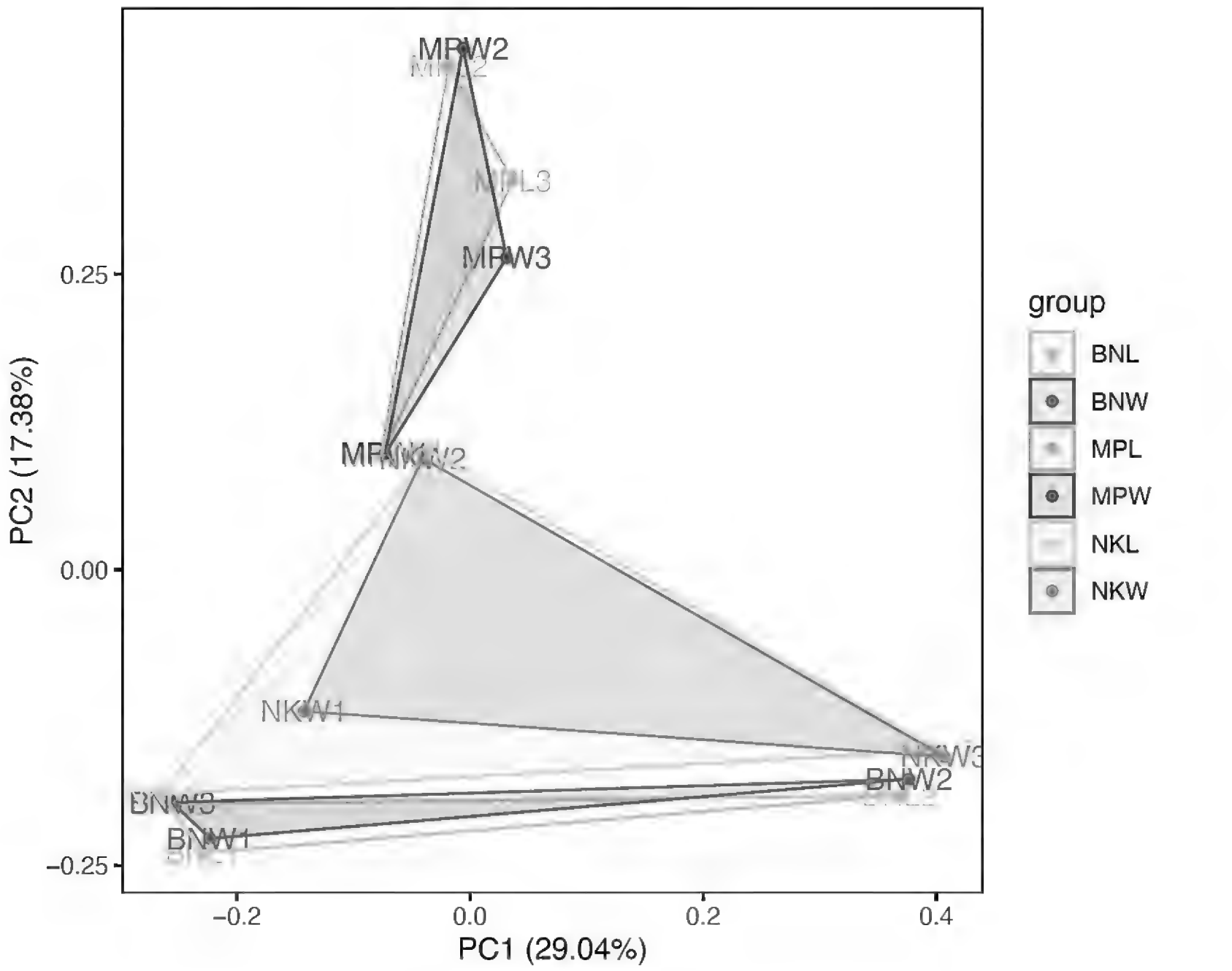


**Figure 7.** Principal coordinate analysis (PCoA), based on Bray-Curtis dissimilarity of microalgae communities at three study areas in Nakhon Si Thammarat, Thailand. MP, BN and NK are the names of the sampling areas. "L" represents larval gut and "W" represents water from breeding sites.

2019; Prasertsin et al. 2021; Ruen-Pham et al. 2021; Limrujiwat et al. 2022; Ruangsomboon and Chonudomkul 2022; Ungwiwatkul and Chantarasiri 2022; Saraphol et al. 2024). These microalgae can be dispersed through natural mechanisms like wind and water currents, as well as by biotic vectors, such as birds, insects and other animals that inadvertently transport microalgae on their bodies or in their digestive tracts. Additionally, human activities and technological advancements have introduced methods for controlled dispersal in both natural and artificial settings (Tesson et al. 2016). However, four taxa were identifiable by both techniques, including *Tetradesmus*, *Nannochloropsis*, *Leptolyngbya* and *Monoraphidium.* This suggested that both techniques exhibited their rigour, where the metabarcoding technique enhanced the capability of capturing the diversity of the microalgae from environmental sources, as suggested by Linne von Berg et al. (2024).

Still, both microscopy and metabarcoding for microalgae identification have the limitation of not being able to identify microalgae down to the species level. While light microscopy is a fundamental tool for microalgae identification, its reliance on morphological characteristics can be a limitation, especially when dealing with species that are cryptic species or very similar genera like *Scenedesmus* versus *Tetradesmus.* Additionally, different life cycle stages of microalgae can exhibit similar morphologies, which can make classification challenging. This is because some stages, like dormant or planktonic forms,

may have similar characteristics despite belonging to different species or even genera that can be mistaken for accurate classification (Figueroa et al. 2018).

For metabarcoding, specific technical limitations in the metabarcoding pipeline or biological characteristics that prevent genus-level taxonomic resolution in this analysis could be attributed to a combination of factors. Primarily, insufficient sequence variation within the target taxa, such as Achnanthales, may prevent precise taxonomic assignment. Lower taxonomic units can only be classified when distinct differences are present in the amplicon sequences. This challenge stems from the inherent nature of conserved versus variable regions within universal gene amplicons. Specifically, the variable regions within a short amplicon often lack enough unique sequence variation to distinguish between closely-related genera or species. This can be due to: (1) low evolutionary rates, i.e. the genes in question may not evolve quickly enough to accumulate sufficient diagnostic mutations for fine-scale differentiation and (2) overlap of intra- vs. inter-genus variation, i.e. in some instances, the genetic variation within a genus can be as high as, or even higher than, the variation between different genera, blurring taxonomic boundaries. Furthermore, this issue is often exacerbated by short amplicon lengths, which inherently limit the capture of sufficient diagnostic sites for genus-level resolution. Finally, the completeness and quality of reference databases also play a critical role; gaps or inaccuracies in these databases can hinder accurate classification.

This study investigated the diversity of algae, a non-monophyletic group of photosynthetic organisms originating from various evolutionary lineages. Consequently, the optimal approach for assessing their diversity involves universal metabarcoding markers such as 18S and 23S ribosomal DNA (rDNA). In this study, we employed 23S rDNA because it is present in plastid genomes and their ancestral cyanobacterial genomes. This allows 23S rDNA to capture the diversity of most photosynthetic organisms, in contrast to 18S rDNA, which is limited to eukaryotic lineages. Although *rbcL* is another frequently used gene for assessing land plant diversity, its utility for universal metabarcoding is limited. This is primarily because the *rbcL* gene is less conserved than ribosomal genes, making it more suitable for targeting known taxonomic groups rather than providing broad coverage of universal organisms (Newmaster et al. 2006).

The specific composition of the mosquito larval diet is influenced by the environment of the breeding site. Factors like the type of soil, water quality, presence of plant matter and abundance of microorganisms can affect the nutritional content of the food available. Mosquito larvae primarily feed on organic matter and microorganisms like bacteria, protozoa, plant debris and insect exuviae found in their breeding sites, with a notable reliance on microalgae for protein (Souza et al. 2019). Microalgae are rich in protein and lipids. They also contain ω-3 polyunsaturated fatty acids, carotenoids, vitamins and β-glucan (review by Ma and Hu (2024)). The protein content in microalgae can vary depending on species and environmental conditions they are grown in.

In the current study, two algal genera were present in all gut and water samples. First is *Tetradesmus* species, a green microalgal genus belonging to the class Chlorophyceae. Species of this genus are primarily found in freshwater environments and are easily recognised due to their coenobial colony, often arranged in tetrads (groups of four cells). They are known as primary producers of wide aquatic ecosystems (Cho and Lee 2024). It is likely that *Tetradesmus*

species could potentially serve as a food source for the mosquito larvae, although a previous study suggested that the larvae of *Culex*, *Aedes* and *Anopheles* did not develop properly in waters containing *Tetradesmus* (Marten 1986). The other genus present in all gut and water samples was the filamentous cyanobacterium *Leptolyngbya*. The predominance of this alga in this study is consistent with results from previous studies that have shown its wide distribution and capability to thrive in diverse environments (Paerl et al. 2000; Gaysina et al. 2019; Nag Dasgupta et al. 2020). However, to date, no information regarding the effects of *Leptolyngbya* toxins on mosquito larvae has been elucidated.

The presence of *Nannochloropsis* in freshwater habitats in this study was congruent with results from Suda et al. (2002). In a previous study, *N. oceanica* microalga has demonstrated effective larvicidal activity against *Culex quinquefasciatus* with 100% mortality within 7 days after feeding on *N. oceanica* cells (Rai and Rajashekhar 2015). Further studies of the genus *Nannochloropsis* are still needed since factors, such as the specific microalgae species, environmental conditions and mosquito species, influence the effects of microalgae on mosquitoes (Tufan-Cetin and Cetin 2023). A previous study by Souza et al. (2019) on *Monoraphidium*, a genus of green microalgae has found that *Ae. aegypti* larvae given microalgae diets have delayed growth rates, prolonged pupation periods, reduced adult emergence and lower survivability. Thus, species-level identification of *Monoraphidium* microalgae is required to determine which ones may affect or inhibit mosquito larvae growth in order to develop an eco-friendly larvicide.

The other two abundant microalgae found in the present study were *Choricystis* and *Parachlorella*. *Choricystis* is a unicellular green alga found in diverse habitats, contributing to nutrient cycling, biofuel production and wastewater treatment through lipid accumulation (Sobczuk and Chisti 2010). *Parachlorella*, a unicellular green microalga, is well-recognised for its high lipid content, making it a promising candidate for biofuel production (Elshobary et al. 2022). Currently, no research has documented the effects of *Choricystis* and *Parachlorella* on mosquito larval development or its insecticide properties. Further studies would be necessary to determine its potential larvicidal properties. While a microalga may show promising larvicidal effects in the laboratory, its success in natural habitats depends on a complex interplay of environmental, biotic and behavioural factors. Environmental fluctuations such as pH, light and nutrients may limit algal viability and metabolite activity in the field. Mosquito larvae may consume a mix of decaying organic matter or other microorganisms, potentially diluting the toxic effects of the microalgal species.

Mosquito larvae are filter feeders using brush filaments in their mouthparts to grasp organic debris and microorganisms from the water in the breeding sites (Rashed and Mulla 1990). They are not selective in what they feed on. However, the size and complexity of the food particles they can ingest tend to increase as they develop through the instars. During development of *Ae. aegypti* larvae from the first to the fourth instar, the mouth brushes are the most evident transformations. The number of lateral palatal brush filaments on the mouth brushes of the first and second instar larvae is less than that of the third and fourth instars (Schaper and Hernández-Chavarría 2006). The amount of these filaments may be associated with a change in diet throughout larval maturation, i.e. the first and second instar larvae feed on bacteria and small food particles, whereas the third and fourth instars consume large organic particles (Rashed and Mulla 1990).

Due to the morphological changes and size of the mouthparts of each instar, there is an expectation of a difference between the microalgae community in their gut and the one in their breeding water. However, in the current study, only pooled third and fourth instar larvae were studied. Spearman's correlation analysis revealed that the microalgal communities in the gut of the third and fourth instar *Ae. aegypti* larvae and their breeding sites were highly correlated, suggesting the larvae consume a variety of the microalgae available in their breeding sites. The diversity of microalgae OTUs found in the gut and breeding sites was not significantly different — both alpha and beta diversity indices suggest that the algal communities were not different across all samples in this study. No ontogenetic dietary shifts across different larval instars were analysed in this study. It is interesting to investigate ontogenetic dietary shifts across different larval instars in the future. Since results from those studies would be valuable and might provide insights into the feeding mechanism, this could be linked to the potential effectiveness of microalgae control agents.

Our results highlighted the strong connections within the microalgal community in both the gut of larvae and breeding sites suggesting that *Ae. aegypti* larvae were capable of consuming almost all the microalgae in their breeding environment. An ideal microalgae-based biocontrol agent should be formulated with a focus on maximising efficacy, stability and ease of application, while minimising environmental impact. Based on our findings, the microalgal genera identified as food sources for *Ae. aegypti* larvae offer potential for developing eco-friendly biocontrol agents. For example, in microalgal strains that can be toxic or produce compounds to interfere with larval development, extract-based formulations should be selected to obtain bioactive compounds. The compounds can be encapsulated in protective materials (e.g. polymers) to enhance stability and controlled release. Additionally, the preferably edible microalgae could be used to deliver targeted control agents through RNAi technology, such as studies reported by Fei et al. (2020, 2021). Moreover, some strains might have volatiles that could be used to induce engorged females to lay eggs in breeding sites or artificial containers. A combination of these microalgae strains or volatile chemical extracts could be used in the oviposition trap.

The selection of microalgal genera should emphasise those with established larvicidal effectiveness or negative impacts on mosquito development, as well as their ecological significance and possibility for large-scale culture for long-term production. Additionally, bioproducts could be delivered directly into mosquito breeding sites. Furthermore, the application method would need to ensure sufficient concentration and contact time for effectiveness. Altogether, the information obtained in this study would be useful in the selection of a strain of microalgae to further investigate for potential application as a new biological control agent for mosquito larvae.

## Conclusions

In this study, ten known genera of microalgae were discovered using light microscopy. Additionally, 25 microalgal genera and an order of diatoms, Achnanthales, were identified using metabarcoding. Spearman's correlation, alpha and beta diversity results revealed a high degree of compositional similarity between gut samples and water samples collected from breeding sites.

The results provided in this study would be useful for the selection of suitable strains of microalgae for further study to develop biocontrol agents, eco-friendly products for the control of mosquito larvae in the future.

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## Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

### Ethical statement

This study was conducted using protocols approved by the animal research ethics committee of Chulalongkorn University Animal Care and Use Protocol (CU-ACUP) (COA No. 014/2565), Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

### Use of AI

No use of AI was reported.

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### Author contributions

Conceptualisation: NJ. Data curation: NJ, JP, NS, EK, SS, PK. Formal analysis: NJ, JP, NS, EK, AR. Investigation: JP, SK, CM. Methodology: NJ, NS, EK, PK. Supervision: NJ, PS, KP, AR. Visualisation: JP, NS, NJ. Writing-original draft: NJ, BKS, EK, NS, PK, JP. Writing-review and editing: NJ, BKS, EK, NS, AR, KP, PS, CW. Funding Acquisition: NJ.

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## Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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## Supplementary material 1

### The study location in map

Authors: Jeerisuda Promprao, Nuttha Sanevas, Ekaphan Kraichak, Santi Saraphol, Piriya Klankeo, Saowalak Kaewmee, Chonlada Mano, Baby Kyi Soe, Anchittha Rujeerapaiboon, Kanok Preativatanyou, Padet Siriyasatien, Catherine Walton, Narissara Jariyapan

Data type: docx

Explanation note: Study locations in Nakhon Si Thammarat Province, Thailand.

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Link: https://doi.org/10.3897/mbmg.9.152948.suppl1

## Supplementary material 2

### BioSample code of the raw data of Sample ID

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Data type: docx

Explanation note: BioSample code of the raw data of Sample ID deposited in NCBI SRA BioProject PRJNA1182502 under Submission ID SUB14847725.

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Link: https://doi.org/10.3897/mbmg.9.152948.suppl2

## Supplementary material 3

### Relative sequence abundance of microalgal taxa

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Data type: docx

Explanation note: Relative sequence abundance of microalgal taxa in the gut of *Ae. aegypti* larvae and water from breeding sites. MP, BN and NK are the names of the sampling areas. "L" represents the larval gut and "W" represents the water from breeding sites.

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Link: https://doi.org/10.3897/mbmg.9.152948.suppl3

## Supplementary material 4

### The average value of alpha diversity indices

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Data type: docx

Explanation note: The average value of alpha diversity indices including Shannon's Index, Simpson's Index, Chao1 and OTU richness, of microalgae in the larval gut compared to those in the water from the same breeding sites.

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## Supplementary material 5

### Pairwise compositional differences

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Data type: docx

Explanation note: Pairwise compositional differences amongst the samples using the Bray-Curtis distance.

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